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# *In vitro* effects of polychlorinated biphenyls on human platelets

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## SUMMARY

Incubation of human platelets with polychlorinated biphenyls (PCB) induced and modulated cellular responses to a different degree. 3,3′,4,4′-tetrachlorobiphenyl (TCB) was a more potent inducer of platelet aggregation, serotonin release and 12-HETE generation compared to the other PCB [2,2′,3,3′-TCB, 3,3′-dichlorobiphenyl (DCB), 2,2′,4,5,5′-pentachlorobiphenyl (PCB)]. 3,3′,4,4′-TCB showed synergistic effects, in combination with other PCB, such as an enhanced formation of 12-HETE, when 3,3′-DCB and 2,2′,3,3′-TCB were applied simultaneously. The combined incubation of platelets with PCB and sodium fluoride (NaF), an activator of G-proteins, resulted in synergistic 12-HETE generation compared to stimulation with NaF or PCB alone. Furthermore, when platelets were incubated with the PCB the enzymatic steps controlling the metabolism of the platelet-activating factor (PAF) were modulated. A direct relationship between the extent of platelet activation and the chloro-substitution pattern of PCB exists.

## INTRODUCTION

Environmental agents of various origin interfere with the cellular components of host defence. They may lead to an activation of immunological and non-immunological mechanisms. Polychlorinated biphenyls (PCB) are a class of ubiquitous environmental contaminants, which are used as hydraulic fluids, adhesives, heat-transfer fluids, flame retardants and dielectric fluids.[1] They are a mixture of chlorinated biphenyls congeners; theoretically 209 of these congeners are possible.[2] PCB are poisonously inert chemicals; due to their stability and lipophilicity, they accumulate in the environment and within the organism. In addition to their carcinogenic potential, these compounds also elicit numerous toxic effects, including immunosuppression with thymic atrophy, oedema, hyperkeratosis, hepatotoxicity and lethality.[3] PCB also induce the activity of the hepatic mixed function oxidase system. Many of the effects have been observed after exposure with complex commercial mixtures of many individual isomers and congeners. Studies with single components have indicated in various systems a correlation with the percentage of the chlorine content as well as the substitution pattern.[4]

Abbreviations: 3,3′-DCB, 3,3′-dichlorobiphenyl; 12-HETE, 12-hydroxyeicosa-6,8,11,14-tetraenoic acid; NaF, sodium fluoride; PAF, platelet-activating factor; PCB, polychlorinated biphenyl; 2,2′,4,5,5′-PCB, 2,2′,4,5,5′-pentachlorobiphenyl; RP-HPLC, reversed-phase high-performance liquid chromatography; 3,3′,4,4′-TCB, 3,3′,4,4′-tetrachlorobiphenyl; 2,2′,3,3′-TCB, 2,2′,3,3′-tetrachlorobiphenyl.

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Platelets play a vital role in haemostasis and in thrombosis.[5] They respond to a wide variety of stimuli and change their shape, release the contents of their granules (e.g. serotonin) and, in the presence of calcium ions, aggregate. They also produce and metabolize bioactive mediators such as 12-hydroxyeicosatetraenoic acid (12-HETE) and the platelet-activating factor (PAF).[6]

It was the purpose of the present study to analyse the effects of four different PCB congeners with regard to different platelet responses, such as aggregation, serotonin-release, PAF metabolization and 12-HETE generation.

## MATERIALS & METHODS

Reagents used were from the following sources: thrombin (from human plasma, 1380 NIH U/mg protein), Ca-ionophore A23187 and sodium fluoride (NaF) were obtained from Sigma, Deisenhofen, Germany. Methanol and chloroform (analytical grade) were from Riedel de Häen, Seelze, Germany. [$^3$H]PAF (alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, 1-0-(alkyl-1′,2′-$^3$H) (specific activity 1·11–2·22 TBq/mmol) and [$^3$H]lyso-PAF (alkyl-sn-glycerol-3-phosphorylcholine, 1-0-(alkyl-1′,2′-[$^3$H]) (1·11–2·22 TBq/mmol specific activity) were supplied by NEN England Nuclear (NEN, Dreieich, Germany).

The polychlorinated biphenyls were purchased from Promochem, Wesel, Germany. They were dissolved in methanol and 10 $\mu$l of the methanolic solution were added to the cell suspension (700 $\mu$l). The following maximal concentrations were tested: 3,3′-DCB, 1 mg/l; 2,2′,3,3′-TCB, 0·034 mg/l; 3,3′,4,4′-PCB, 0·175 mg/l, 2,2′,4,5,5′-PCB, 0·031 mg/l. The various concentrations resulted from the different water solu-

bility of the PCB. The effects of 0·6 μM of the test compound were shown.

*Preparation of the platelets*

Platelets were isolated from platelet-rich plasma (PRP). PRP was obtained by centrifugation of peripheral blood (9 ml) supplemented with phosphate-buffered saline (PBS) containing 1·5% EDTA (1 ml) at 200 *g* (25 min at 20°). The supernatant was mixed with an equal volume of PBS with 1·5% EDTA and centrifuged at 1285 *g* (20 min at 4°). The pellet was washed in 10 ml PBS with 1·5% EDTA. After centrifugation the platelets were resuspended to a final concentration of $2 \times 10^8$ cells/ml in PBS.

*Stimulation of the platelets and analysis of mono-HETE*

Platelets ($1 \times 10^8$) were incubated in the presence of calcium (1 mM) and magnesium (0·5 mM) with the indicated stimuli (PCB, methanol and/or NaF) for 20 min at 37°. The reaction was terminated by the addition of methanol/acetonitril (50:50, v/v) and centrifugation at 1900 *g* for 15 min at 4°. The supernatants were evaporated by lyophilization, the remainder was dissolved in methanol/water (30:70, v/v) and subjected to RP-HPLC analysis. Rapid analysis of mono-HETE by RP-HPLC was determined as previously described[7] with a solvent mixture of phosphate buffer (6 mM dipotassium hydrogenphosphated, containing 0·05% EDTA, adjusted to pH 5·0 with phosphoric acid), acetonitrile and methanol (28:42:30, v/v). The effluent was monitored at 235 nm.

*Determination of PAF metabolism*

Human platelets ($1 \times 10^8$) were preincubated for 2 min with 0·74 kBq [$^3$H]PAF or [$^3$H]lyso-PAF in the presence of calcium (1 mM) and magnesium (0·5 mM). Subsequently, stimulation was performed over 20 min with the various stimuli (PBS, methanol, PCB and the Ca-ionophore). The incubation was stopped by adding 2 ml of chloroform:methanol (2:1) and after subsequent centrifugation for 10 min at 4000 r.p.m. The resulting methanol-water-phase was extracted with chloroform and the combined chloroform extracts were dried. The residues were spotted on a silica gel thin-layer plate (250 μm, Kieselgel 60, Merck, Darmstadt, Germany). As mobile phase, served a mixture of chloroform-methanol-water-acetic acid (50 25:4:8, v/v). Radioactivity was detected with an ISOMESS Radiodünnschicht-Analysator IM 3000 (Straubenhardt, Germany). The typical rf-values were for lyso-PAF 0·31, for PAF 0·41 and for alkyl-acyl-GPC 0·7.

*Release of [$^3$H]serotonin*

The release of serotonin [1,2-$^3$H(N)-5-Hydroxytryptamine 0·7 TBq/mmol] was determined as previously described.[8] Briefly, human platelets were incubated with [$^3$H]serotonin (7·5 kBq/$10^8$ cells) for 30 min at 37°. During the incubation 65–75% of the radioactivity was incorporated. After washing twice with PBS the labelled platelet suspension was incubated with PCB, methanol (as control), PBS, thrombin or Ca-ionophore A23187 for 20 min at 37°. The release of [$^3$H]serotonin was determined after mixing the platelet suspension with 0·5 ml of 3% paraformaldehyde in PBS. After centrifugation the supernatant was analysed for radioactivity by liquid scintillation counting.

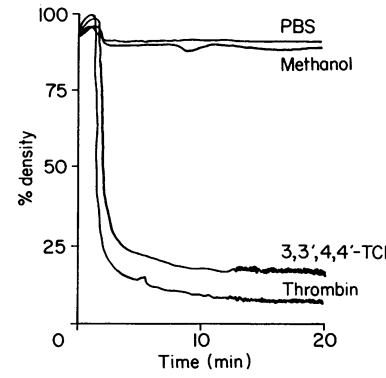


**Figure 1.** Effects of 3,3',4,4'-TCB on platelet aggregation. Platelets ($1 \times 10^8$) were incubated with 3,3',4,4'-TCB (0·6 μM), thrombin (2·5 U/ml), PBS or methanol (10 μl/700 μl) for 20 min at 37°. Aggregation was recorded continuously. Aggregation curves demonstrated are representative of at least five experiments.

## RESULTS

Stimulation of human platelets ($2 \times 10^8$/ml) in the presence of calcium (1 mM) and magnesium (0·5 mM) with thrombin (2·5 U/ml) caused aggregation, as was recorded by the change in light transmission. A comparable aggregation was observed when platelets were incubated in the presence of calcium and magnesium with 3,3',4,4'-TCB (0·6 μM) (Fig. 1). The aggregation peak induced by 3,3',4,4'-TCB reached about 80% of the thrombin-induced aggregation. The remaining compounds (3,3'-DCB, 2,2',3,3'-TCB and 2,2',4,5,5'-PCB), at various concentrations, PBS or the addition of methanol (10 μl as control) did not induce platelet aggregation.

Then we examined the capacity of PCB to stimulate the release of serotonin. For this purpose [$^3$H]serotonin-labelled platelets were incubated with various concentrations of the indicated PCB or the same volume of methanol (as control) for 20 min at 37°. Incubation of platelets with 3,3',4,4'-TCB increased the release of serotonin compared to the control (in the absence of PCB but in the presence of methanol). A comparison of the different PCB showed that 3,3',4,4'-TCB was the most potent PCB. About 25–30% of the total radioactivity was obtained in the supernatant. Ca-ionophore or thrombin as stimuli led to a release which amounted to 75% or 90%, respectively; thus, 3,3',4,4'-TCB is only a weak secretagogue for serotonin. Serotonin release induced by thrombin or Ca-ionophore A23187 was not significantly affected in the presence of 3,3',4,4'-TCB (data not shown). Similar results were obtained when the PCB were diluted in PBS instead of methanol.

In addition to the serotonin release, platelets were able to generate and metabolize newly generated mediators, such as the 12-lipo-oxygenase-products (12-HETE) and the platelet-activating factor (PAF).

In order to analyse the metabolism of PAF, platelets ($1 \times 10^8$) were preincubated for 2 min at 37° with [$^3$H]PAF (0·74 kBq); subsequently the various PCB, the same volume of methanol (as control) or the Ca-ionophore (5 μM) were added and incubation proceeded for additional 20 min. Our data demonstrate (Fig. 2a) that platelets metabolize exogenously added PAF into lyso-PAF and alkyl-acyl-GPC. After 20 min of incubation in the presence of the Ca-ionophore more than 75% of PAF was metabolized and alkyl-acyl-GPC was the main

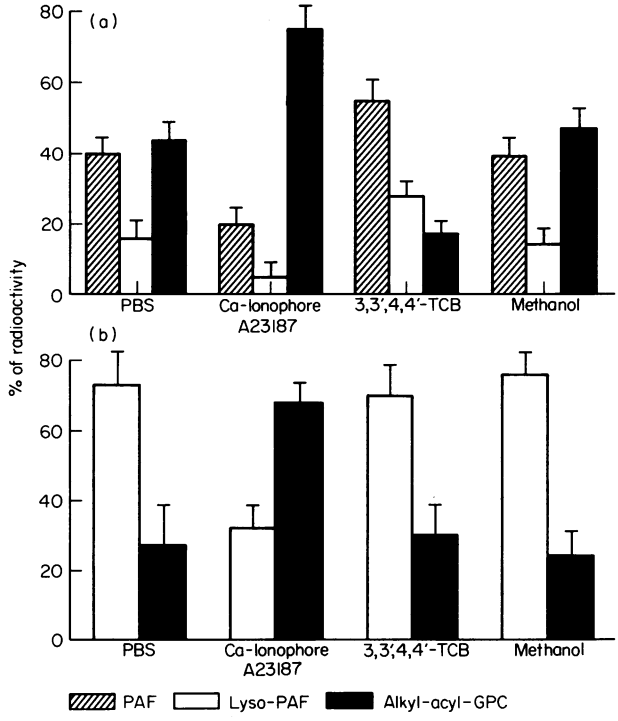


**Figure 2.** Effect of PCB on the metabolism of PAF (a) and lyso-PAF (b). Platelets were preincubated with [$^3$H]PAF or [$^3$H]lyso-PAF; subsequently the indicated stimuli were added and the incubation proceeded for additional 20 min. Each value represents the mean ± SD of five (a) or three (b) independent experiments with different donor cells.

metabolite; only minute amounts of lyso-PAF were detected. Platelets incubated with methanol (10 μl) metabolized 60% of PAF into lyso-PAF (20%) and alkyl-acyl-GPC (40%). In contrast, the incubation of the platelets with 3,3',4,4'-TCB decreased the metabolism of PAF; only 40% of the PAF was metabolized into lyso-PAF and alkyl-acyl-GPC. Similar effects were observed when the platelets were incubated with the remainder PCB.

Experiments were carried out to study the metabolism of lyso-PAF. Platelets were able to metabolize lyso-PAF to alkyl-acyl-GPC. No PAF was detectable. Our data demonstrate (Fig. 2b) that platelets in the presence of the Ca-ionophore A23187 metabolize 60% of the lyso-PAF to alkyl-acyl-GPC. In contrast, in the presence of 3,3',4,4'-TCB or methanol as control about 30% of the lyso-PAF was metabolized.

Experiments were then performed to analyse the effects of PCB incubation on the 12-HETE formation in platelets. Human platelets incubated with stimuli such as the Ca-ionophore, thrombin and arachidonic acid generated 12-HETE in a time- and dose-dependent manner. The cells ($1 \times 10^8$) were incubated with either the indicated PCB or a combination of two PCB or the same volume of methanol (as control) for 20 min at 37° (Fig. 3). As is apparent, the generation of 12-HETE was significantly enhanced when the platelets were stimulated with 3,3',4,4'-TCB. In contrast, 3,3'-DCB, 2,2',3,3'-TCB and 2,2',4,5,5'-PCB were not effective. The combination of 3,3'-DCB or 2,2',3,3'-TCB with 3,3'4,4'-TCB resulted in a synergistic effect on 12-HETE generation. The incubation of platelets with 3,3',4,4'-TCB in combination with 2,2',4,5,5'-PCB led to a

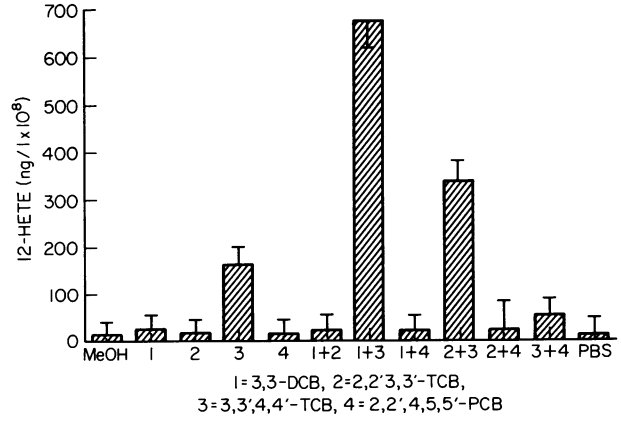


**Figure 3.** Induction of 12-HETE generation from human platelets by PCB. Platelets ($1 \times 10^8$) were incubated PCB (0·6 μM) for 20 min at 37°. 12-HETE generation was measured by RP-HPLC. The data are expressed as mean ± SD of three independent experiments.

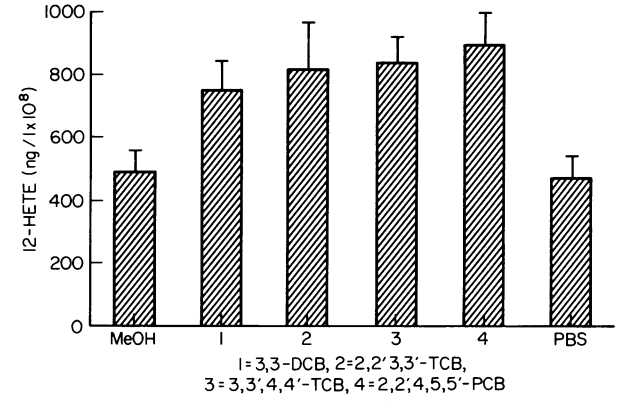


**Figure 4.** Modulatory effect of PCB on 12-HETE generation from NaF (20 mM)-stimulated platelets. 12-HETE generation was induced by the simultaneous addition of NaF and the indicated PCB (0·6 μM). The data were calculated as means ± SD of three independent experiments.

reduction of 12-HETE compared to the potency of 3,3',4,4'-TCB alone. No significant changes were observed when the other PCB were combined, as indicated.

In previous publications[9] we have demonstrated that fluoride ions (NaF) are potent stimuli for 12-HETE generation from platelets due to their activation of G-proteins. We then studied the modulatory influence of the PCB on 12-HETE generation induced by stimulation with NaF. Platelets were incubated simultaneously with NaF (20 mM) and the respective PCB for 20 min at 37°. The incubation of NaF in the presence of the same volume methanol instead of PCB served as control. As is presented in Fig. 4, the addition of NaF and PCB showed an enhancing effect of 12-HETE generation compared to NaF stimulation in the absence of PCB. A synergistic effect was obtained in combination with NaF and the PCB.

## DISCUSSION

It has been demonstrated in the past that pesticides, such as dieldrin, DDT, heptachlor, induce the release of histamine from

rat mast cells and human basophils;[10] furthermore incubation of macrophages with polychlorinated cyclic hydrocarbons (y-HCH) led to the release of lipo-oxygenase products.[11]

Our data clearly demonstrate that PCB initiate and/or modulate cellular responses of human platelets. 3,3'4,4'-TCB, a PCB congener with chloro-substitution in two para and in two meta positions, is more potent in platelet aggregation, serotonin release and 12-HETE generation compared to other PCB. It is well-known that PCB without chloro-substitution in the ortho-position and a high degree in co-planar conformation, such as 3,3',4,4'-TCB, induce a defined pattern of drug-metabolizing enzymes.[12] Our data show a direct relationship between the extent of platelet stimulation and the chloro-substitution pattern (3,3',4,4'-TCB versus 2,2',4,4'-TCB).

Incubation of the platelets with the PCB decreased the metabolism of exogenously added [$^3$H]PAF to [$^3$H]lyso-PAF and [$^3$H]alkyl-acyl-GPC. No significant differences were observed when the lyso-PAF metabolism to alkyl-acyl-GPC was analysed in the presence of methanol or 3,3',4,4'-TCB. Thus, the data suggest that the metabolism of lyso-PAF to alkyl-acyl-GPC catalysed by acyltransferase is not affected in the presence of PCB compared to the control. In conclusion our data with regard to the metabolism of PAF and lyso-PAF suggest that incubation of platelets with PCB affects the first enzymatic step, the metabolism of PAF to lyso-PAF. One may suggest that the activity of the enzyme acetyl-hydrolase is impaired.

Platelet activation which results in platelet responses is transmitted via a complex signal transduction pathway. Due to their high lipophilic character PCB interact with membranes, but the precise mechanisms of the PCB–cell interaction and the subsequent stimulation of the signal transduction sequence are not known. In a previous report it has been shown that PCB have effects on protein kinase C (PKC) activity obtained from rat and mouse brain.[13] Preliminary experiments as to the effects of PCB on platelet PKC activity (measured as described by König *et al.*[14] demonstrated that incubation of platelets with methanol or PCB led to a loss of PKC from the cytosol compared to untreated cells (data not shown). The disappearance of PKC from the cytosol may provide indirect evidence for its activation and translocation.

Furthermore, G-proteins are involved in the cellular responses of platelets.[5,9,14] Our experiments demonstrate a synergistic effect on 12-HETE generation with the combination of PCB and NaF. The enhanced 12-HETE-generation in the presence of a G-protein activator (NaF) and PCB suggests that more than one signal transduction pathway may be involved in 12-HETE generation.

Further experiments are needed to clarify which components of the signal transduction pathway (G-protein activation, PKC translocation, phosphoinositol turnover and/or rise in internal calcium concentration) are directly involved and affected after stimulation of platelets with PCB.

*In vitro* data may have relevance for the immunological alterations observed *in vivo*. In fact the mean PCB serum levels are about 5–7 ng/ml or higher without any documented unusual PCB exposure.[1] Sahl *et al.*[15] determined PCB blood levels of personnel from an electric utility between 1 and 37 μg/l. In comparison, the *in vivo* PCB concentrations in blood after exposure are higher than the PCB concentrations in our study. One has to emphasize that PCB commercially used are complex mixtures of many isomers and that the PCB serum concentration comprise the combined amounts of all detectable PCB within serum.

Our data thus emphasize that PCB affect platelet function *in vitro* by concentrations which correlate with PCB blood levels obtained after exposure *in vivo*.

## ACKNOWLEDGMENT

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